5-Methyltetrahydrofolate, the Active Form of Folic Acid, Restores Endothelial Function in Familial Hypercholesterolemia

Marianne C. Verhaar, MD; Robert M.F. Wever, Pharm D; John J.P. Kastelein, MD, PhD; Thea van Dam; Hein A. Koomans, MD, PhD; Ton J. Rabelink, MD, PhD

Background—Impaired nitric oxide (NO) activity is an early event in the pathogenesis of cardiovascular disease, resulting from either reduced NO formation or increased NO degradation. Administration of tetrahydrobiopterin (BH4), an essential cofactor for NO production, could restore NO activity in familial hypercholesterolemia (FH). Because folates have been suggested to stimulate endogenous BH4 regeneration, we hypothesized that administration of 5-methyltetrahydrofolate (5-MTHF, the active circulating form of folate) might improve NO formation in FH.

Methods and Results—We studied the effects of 5-MTHF on NO bioavailability in vivo in 10 patients with FH and 10 matched control subjects by venous occlusion plethysmography, using serotonin and nitroprusside as endothelium-dependent and -independent vasodilators. In vitro, we investigated the effect of 5-MTHF on NO production by recombinant endothelial NO synthase (eNOS) by use of [3H]arginine to [3H]citrulline conversion. We also studied the effects of 5-MTHF on superoxide generation by eNOS and xanthine oxidase (XO) by use of lucigenin chemiluminescence. The impaired endothelium-dependent vasodilation in FH (63% versus 90% in control subjects) could be reversed by coinfusion of 5-MTHF (117% vasodilation), whereas 5-MTHF had no significant effect on endothelium-dependent vasodilation in control subjects. 5-MTHF did not influence basal forearm vasomotion or endothelium-independent vasodilation. 5-MTHF had no direct effect on in vitro NO production by eNOS. However, we did observe a dose-dependent reduction in both eNOS- and XO-induced superoxide generation.

Conclusions—These results show that the active form of folic acid restores in vivo endothelial function in FH. It is suggested from our in vitro experiments that this effect is due to reduced catabolism of NO. (Circulation. 1998;97:237-241.)

Key Words: endothelium ■ endothelium-derived factors ■ hypercholesterolemia ■ folates

Impaired vascular NO activity has emerged as an early marker for cardiovascular disease. Indeed, most risk factors for atherosclerosis have been shown to be associated with impaired endothelium-dependent vasodilatation because of reduced NO availability. The precise mechanism responsible for this reduced NO availability is unknown; both impaired formation and increased degradation of NO may be involved.

NO formation is critically dependent on the presence of the cofactor BH4, which stimulates conversion of L-arginine to L-citrulline and NO by NO synthase.1,2 BH4 acts as a cofactor by providing electrons, thus being oxidized to the inactive qBH2.3 We recently demonstrated that administration of BH4 could restore impaired NO activity in hypercholesterolemia.4 This would suggest a role for BH4 as a therapy to increase the antithromogenic potential of the endothelium. However, BH4 is active only in its (unstable) reduced form and thus not suitable for oral supplementation. Folates have been suggested to stimulate endogenous BH4 regeneration from qBH2.5,6 One could therefore hypothesize that administration of folates may increase NO formation.

If true, such a role of folates would be of major clinical significance, because oral folic acid administration could be explored as a cheap and safe therapy to reduce cardiovascular risk. To evaluate this hypothesis, we studied the effects of 5-MTHF, the active form of folic acid, on endothelium-dependent and -independent vasodilation in hypercholesterolemic patients and control subjects. In addition, to elucidate possible mechanisms, we investigated the effect of 5-MTHF on NO availability in vitro.

Methods

Effect of 5-MTHF on NO Availability In Vivo

Subjects

Ten patients with FH and 10 healthy control subjects, matched for age, sex, and smoking habit, participated in our study. The diagnosis

Received September 16, 1997; revision received November 10, 1997; accepted November 13, 1997.

From the Departments of Nephrology and Hypertension (M.C.V., T v D., H.A.K., T.J.R.) and Clinical Chemistry (R.M.F.W.), University Hospital Utrecht, and Department of Vascular Medicine (J.J.P.K.), University Medical Centre Amsterdam, Netherlands.

Dr Verhaar and Wever contributed equally to this study.

Correspondence to Ton J. Rabelink, Department of Nephrology and Hypertension, University Hospital Utrecht, Heidelberglaan 100, PO Box 85500, 3508 GA Utrecht, The Netherlands.

E-mail T. Rabelink @digd.azu.nl

© 1998 American Heart Association, Inc.
of FH was based on established criteria. In all patients, a molecular diagnosis of FH was confirmed. Studies were performed at least 2 weeks after withdrawal of lipid-lowering medication. None of the participants in our study had clinical signs of cardiovascular disease. They did not use vasoactive medications in the week before the study, and all abstained from alcohol, tobacco, and caffeine-containing drinks for at least 12 hours before measurements were made. All subjects gave written informed consent.

Protocol

The study protocol was approved by the local research ethics committee of the University Hospital Utrecht. All experiments were performed in a quiet room kept at a constant temperature of 22°C to 24.5°C. Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography as described previously.4

For assessment of endothelium-dependent vasodilatation, serotonin (Sigma Chemical Co) was infused into the brachial artery in increasing doses of 0, 0.6, 1.8, and 6.0 ng · 100 mL FAV⁻¹ · min⁻¹ (block A). These dosages have previously been shown to cause NO-mediated vasodilatation.4 For assessment of endothelium-independent vasodilatation, sodium nitroprusside (Merek) was administered intra-arterially at incremental doses of 0, 6, 60, 180, and 600 ng · 100 mL FAV⁻¹ · min⁻¹ (block B). During blocks A and B, saline was coinfused. The order of blocks A and B was randomized. Subsequently, 5-MTHF (Bigmar Pharmaceuticals) was infused at rates of 0, 0.1, 1, and 10 µg · 100 mL FAV⁻¹ · min⁻¹, for 5 minutes per dose, to assess the influence of 5-MTHF on baseline vasomotion. These dosages were chosen to achieve calculated plasma concentrations in the forearm of 0.1, 1, and 10 µmol/L, which have been shown to be biologically active concentrations in vitro.11 Finally, after at least a 15-minute infusion of 5-MTHF at a rate of 1 µg · 100 mL FAV⁻¹ · min⁻¹, the first two infusion blocks (A and B) were repeated in randomized order during 5-MTHF confusion.

Venous blood samples were obtained from the brachial vein before and after 15 to 20 minutes of 5-MTHF infusion into the ipsilateral brachial artery and immediately centrifuged; plasma was stored at −20°C until analysis. Plasma folate and vitamin B₆ were measured by a competitive immunoassay using direct chemiluminescence (CIBA Corning Diagnostics Corp). Plasma total homocysteine was measured by the method described by Araki and Sako.13 Plasma creatinine, total cholesterol, HDL cholesterol, triglyceride, and apolipoprotein B were measured with standard laboratory methods. LDL cholesterol was calculated by the Friedewald formula.

Effect of 5-MTHF on NO Availability In Vivo

Effect of 5-MTHF on NO Production by Recombinant eNOS

Experiments were performed on recombinant eNOS, derived from a baculovirus/Sf9 expression system (kindly donated by Tanya Mogetlivich, Cayman Chemical Co, Ann Arbor, Mich), which shares important features with the native eNOS; it is appropriately targeted to the particulate subcellular fraction and shares cofactor requirements similar to those of eNOS isolated from endothelial cells.12 NO activity was measured as the formation of 1-[2,3,4,5H]citrulline from [1-15N]arginine (Amersham). We investigated the effects of 30 minutes of incubation with 5-MTHF (Sigma Chemical Co; 10, 100, and 1000 µmol/L) on eNOS activity. To exclude the possibility that lack of prosthetically bound qBH₂ would limit regeneration of BH₄, we also investigated the effect of 5-MTHF on NO production with coinucubation of qBH₂ (10 µmol/L). In addition, the effects of BH₄ (10 µmol/L) on eNOS were studied as a positive control. All experiments were performed in triplicate.

Effect of 5-MTHF on Superoxide Generation by eNOS and Xanthine Oxidase/Hypoxanthine

To study the actions of 5-MTHF as an antioxidant, the effects of 5-MTHF (10, 100, and 1000 µmol/L) on superoxide generation by purified recombinant eNOS and xanthine oxidase (Sigma, 4 µL)/hypoxanthine (Sigma, 0.3 mmol/L) were investigated. Both systems are relevant to the increased generation of reactive oxygen species in hypercholesterolemia and atherosclerosis.13,14 Superoxide generation was measured with lucigenin-enhanced chemiluminescence, as described previously. In short, scintillation vials containing lucigenin (250 µmol/L) and eNOS (12 µg) were placed into a Berthold luminometer (AutoLumat LB 953) at 37°C in the presence of 0.5 mmol/L NADPH, 300 U/mL calmodulin, and 1 mmol/L calcium. Counts were recorded for 5 minutes, and the respective backgrounds were subtracted. All measurements were performed in triplicate. Specificity of the chemiluminescence signal for superoxide was controlled by incubation with superoxide dismutase.

Analysis

Average values of forearm blood flow were obtained from the last five or six consecutive recordings of each measurement period. The ratio of flows in the infused and noninfused arms (M/C ratio) was calculated for each time point and expressed as percentage change from baseline. Results of in vivo studies are expressed as mean±SEM. Differences in forearm vascular reactivity induced by 5-MTHF were examined by repeated-measures ANOVA for a randomized block design, where the interaction variance ratio indicates differences between the curves (Jandel Scientific Inc). Group comparisons with respect to clinical characteristics were made with unpaired and two-tailed t tests. Results of in vitro experiments are presented as mean±SEM of three experiments. These data were examined by ANOVA. If variance ratios reached statistical significance, differences between the means were analyzed with the Student-Newman-Keuls test for P<0.05 and P<0.01.

Results

Effect of 5-MTHF on NO Availability In Vivo

Patient characteristics and baseline laboratory data are shown in the Table.

Influence of 5-MTHF on Baseline Hemodynamics and Biochemical Parameters

Cumulative dose infusion of 5-MTHF did not significantly change basal forearm blood flow in either FH patients or control subjects (M/C ratio in patients: 1.15±0.15, 1.10±0.12, 1.09±0.13, and 1.17±0.16 and in control subjects: 1.24±0.11, 1.27±0.15, 1.28±0.17, and 1.26±0.16). There was also no significant effect of 5-MTHF infusion on mean arterial pressure in either group (patients: 79±6 mHg versus 78±2 and control subjects: 81±3 versus 81±2 mm Hg, P=NS). Infusion of 5-MTHF significantly increased folate levels in both patients (9.1±1.0 to 240±60 mmol/L, P<0.05) and control subjects (9.4±1.5 to 307±74 mmol/L, P<0.01) but did not significantly alter homocysteine levels (patients: 8.7±0.7 to 8.7±0.6 and control subjects: 9.1±0.5 to 8.7±0.7 µmol/L, P=NS).

Influence of 5-MTHF on Endothelium-Dependent Vasodilation

In patients, serotonin-induced vasodilation was significantly impaired compared with control subjects (M/C ratio from

Select Abbreviations and Acronyms

BH₄ = tetrahydrobiopterin
eNOS = endothelial NO synthase
FAV = forearm volume
FH = familial hypercholesterolemia
5-MTHF = 5-methyltetrahydrofolate
qBH₂ = quinoid dihydrobiopterin (oxidized BH₄)
1.24±0.10 to 1.98±0.19 versus 1.38±0.16 to 2.62±0.34, P<0.05). 5-MTHF coinfusion significantly enhanced serotonin-induced vasodilation (1.08±0.11 to 2.28±0.21 increase in M/C ratio, P<0.01 versus saline coinfusion), whereas coinfusion of 5-MTHF had no significant effect on serotonin-induced vasodilation in control subjects (M/C ratio from 1.38±0.16 to 2.62±0.34 versus 1.13±0.12 to 2.31±0.27, P=NS). There was no significant difference in endothelium-dependent vasodilation between patients during 5-MTHF coinfusion and control subjects (Fig 1).

Influence of 5-MTHF on Endothelium-Independent Vasodilation

Administration of the endothelium-independent vasodilator sodium nitroprusside caused increases in forearm blood flow (M/C ratio), which were not significantly different between FH patients and control subjects (1.12±0.09 to 6.98±0.65 versus 1.28±0.07 to 6.04±0.69, respectively; P=NS). Coinfusion of 5-MTHF did not significantly alter endothelium-independent vasodilation in FH patients or in control subjects. There was no significant difference in sodium nitroprusside-induced vasodilation between FH patients and control subjects during 5-MTHF infusion (1.02±0.10 to 5.46±0.50 versus 1.08±0.07 to 5.02±0.40, respectively; P=NS) (Fig 1).

Effect of 5-MTHF on NO Availability In Vitro

Effects of 5-MTHF on NO Production by Endothelial Cells and Recombinant eNOS

NO production doubled, from 7.9±0.2 to 17.2±0.3 pmol·min⁻¹·mg protein⁻¹ (P<0.01), after administration of BH₄. Addition of 5-MTHF (10, 100, and 1000 μmol/L) had no effect on NO production by recombinant eNOS (from 7.9±0.2 to 7.1±0.4, 7.2±0.3, and 7.0±0.4 pmol·min⁻¹·mg protein⁻¹, respectively). Also, addition of 5-MTHF in the presence of surplus oxidized BH₄ did not stimulate NO production (6.9±0.4, 7.0±0.3, and 6.1±0.5 pmol·min⁻¹·mg protein⁻¹, respectively; P=NS compared with eNOS alone).

Effects of 5-MTHF on Superoxide Generation by Recombinant eNOS and Xanthine Oxidase/Hypoxanthine

5-MTHF dose-dependently reduced superoxide production by both xanthine oxidase and eNOS (Fig 2). Control experiments with superoxide dismutase confirmed that the observed lucigenin signal in these experiments was superoxide-mediated (data not shown).

Discussion

Administration of 5-MTHF, the active circulating form of folic acid, restores endothelial function in hypercholesterolemic patients without overt macrovascular disease. Serotonin-induced NO activity, which was significantly impaired in our group of hypercholesterolemic patients, could be completely restored by local infusion of 5-MTHF, whereas 5-MTHF had no significant effect on NO activity in healthy control subjects. Infusion of 5-MTHF also did not influence basal forearm vasomotion or endothelium-independent vasorelaxation.

NO bioavailability is a result of both NO production and NO degradation. NO production is catalyzed by eNOS, which requires BH₄ as an essential cofactor. Indeed, our in vitro experiments show that BH₄ increases NO production by eNOS. 5-MTHF has been shown to stimulate reduction of qBH₄ back into the active form BH₄⁻. Our in vitro experiments demonstrate no direct effect of 5-MTHF on NO production by eNOS. However, this does not exclude an

Clinical Characteristics and Laboratory Data

<table>
<thead>
<tr>
<th></th>
<th>FH</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>35±4</td>
<td>35±3</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>6/4</td>
<td>7/3</td>
</tr>
<tr>
<td>Smoking, +/−</td>
<td>1/9</td>
<td>1/9</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.6±0.9</td>
<td>22.6±0.7</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>79±2</td>
<td>81±3</td>
</tr>
<tr>
<td>Basal forearm blood flow, mL·10⁰ mL FAV·min⁻¹</td>
<td>2.6±0.2</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>8.2±0.6*</td>
<td>4.9±0.3</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.45±0.23</td>
<td>1.01±0.13</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.1±0.1*</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>6.5±0.5*</td>
<td>2.9±0.3</td>
</tr>
<tr>
<td>Apolipoprotein B, g/L</td>
<td>1.6±0.1*</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>8.7±0.7</td>
<td>9.1±0.5</td>
</tr>
<tr>
<td>Folate, nmol/L</td>
<td>9.1±1.0</td>
<td>9.4±1.5</td>
</tr>
<tr>
<td>Vitamin B₁₂, pmol/L</td>
<td>226±17</td>
<td>203±25</td>
</tr>
</tbody>
</table>

*P<.05 vs control.

BMI indicates body mass index; MAP, mean arterial pressure. Data are expressed as mean±SEM.

αBH₂ back into the active form BH₄⁻. Our in vitro experiments show that BH₄ increases NO production by eNOS. 5-MTHF has been shown to stimulate reduction of qBH₄ back into the active form BH₄⁻. Our in vitro experiments demonstrate no direct effect of 5-MTHF on NO production by eNOS. However, this does not exclude an

Figure 1. A and C, Percentage change in forearm blood flow after stimulation of endothelium-dependent and endothelium-independent vasodilation with serotonin (5-HT) and sodium nitroprusside (SNP), respectively, in control subjects. B and D, Same parameters for patients with FH.
effect of 5-MTHF on NO production in vivo, in that it may serve as an electron donor to pterin-reducing enzymes. Enhanced oxidative degradation of NO is a major determinant of impaired NO availability in hypercholesterolemia. This may be due to increased eNOS-induced generation of superoxide or elevated circulating levels of xanthine oxidase. In the present study, 5-MTHF caused a dose-dependent reduction in both eNOS- and xanthine oxidase–induced superoxide production, suggesting that 5-MTHF may reverse the derangement in NO metabolism that occurs in hypercholesterolemia by reduction of reactive oxygen species. Our data suggest that this could be a direct antioxidant effect. However, an indirect effect is possible as well, by either improvement of the cellular antioxidant defense system, reduction of the pro-oxidant homocysteine, or an increase in BH4 availability.

The observed improvement in NO activity suggests reduced availability of folate in our hypercholesteremic patients. Plasma folate levels in our subjects were within the normal range, with similar values for patients and control subjects. However, because plasma folate concentrations may not accurately reflect tissue folate stores, we cannot exclude the possibility of reduced intracellular folate levels or altered intracellular folate metabolism in hypercholesterolemia. Folate administration has been shown to decrease homocysteine levels, to lower cardiovascular risk in homocysteinuric patients, and to improve endothelial function (estimated as plasma concentrations of endothelium–derived proteins) in patients with mild to moderate hyperhomocysteinemia. However, this homocysteine-lowering effect is not likely to be involved in our study. Plasma homocysteine levels were not elevated in our population and did not change during 5-MTHF infusion. Furthermore, the increase in NO availability occurred only in patients but not in healthy control subjects, whereas homocysteine levels were similar in both groups.

In conclusion, our data indicate that 5-MTHF can restore endothelial function in hypercholesterolemic patients, probably by affecting cellular oxidative metabolism. This mode of action suggests that the effect of 5-MTHF on NO activity can be extrapolated to other clinical conditions that have been associated with impaired NO activity. Our finding warrants further exploration of the potential of oral folic acid therapy as a novel, safe, and inexpensive tool to reduce cardiovascular risk, not only in hyperhomocysteinemia but also in other risk factors for cardiovascular disease, such as hypercholesterolemia.

Acknowledgments
This study was supported by the Dutch Heart Foundation (grant 96.169). Dr Rabelink is supported by the Royal Dutch Academy of Sciences. We acknowledge the technical advice from Dr K. van Kessel and Dr D. van Loon (Antonius Ziekenhuis, Nieuwegein) for homocysteine determinations.

References
5-Methyltetrahydrofolate, the Active Form of Folic Acid, Restores Endothelial Function in Familial Hypercholesterolemia
Marianne C. Verhaar, Robert M. F. Wever, John J. P. Kastelein, Thea van Dam, Hein A. Koomans and Ton J. Rabelink

*Circulation.* 1998;97:237-241
doi: 10.1161/01.CIR.97.3.237

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/3/237

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at: http://circ.ahajournals.org/subscriptions/